***Pseudo-nitzschia* spp. (Bacillariophyceae) and Dissolved Organic Matter (DOM)
dynamics in the Ebro Delta (Alfacs Bay, NW Mediterranean Sea)**

Running Head: *Pseudo-nitzschia* spp. nutrition and DOM in a coastal embayment

Sofia Loureiro¹, Esther Garcés^{1*}, Margarita Fernández-Tejedor², Dolors Vaqué¹ and Jordi
Camp¹

¹Departament de Biologia Marina i Oceanografia, Institut de Ciències del Mar, ICM – CSIC.

Pg. Marítim de la Barceloneta 37-49, 08003 Barcelona, Catalonia, Spain

²IRTA, Institut de Recerca i Tecnologia Agroalimentaries, 043540 St Carles Ràpita,
Catalonia, Spain

ABSTRACT:

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* corresponding author Email: esther@icm.csic.es, Phone: +34 93 2309613 Fax: +34 93 2309555

INTRODUCTION

The influence of organic nutrients in the development of HABs (Harmful Algal Blooms) has motivated a recent line of research concerning the nutritional strategies of potentially harmful algae species (Jauzein et al., 2008; Stolte et al., 2002). Accordingly, the gradual change in the composition of agricultural fertilizers in favour of organic components (urea now represents > 50% of worldwide nitrogen fertilizer) has been linked to the increment of harmful algae species in the aquatic environment (Glibert et al., 2006). Although toxic dinoflagellates are amply recognized by its mixotrophic abilities (e.g. Adolf et al., 2006; Collos et al., 2007; Legrand and Carlsson, 1998), *Pseudo-nitzschia* spp., a diatom genus that includes species able to produce domoic acid (DA) and thereby Amnesic Shellfish Poisoning (ASP) by ingestion of contaminated vectors (e.g. shellfish, sardines), has also been the focus of investigation as regards organic components. Specifically, *P. multiseries* was found to grow equally well on glutamine and urea as on nitrate (Hillebrand and Sommer, 1996); the same species was suggested to use organic molecules as a dark survival strategy (Mengelt and Prézelin, 2002); *P. australis* was able to use both inorganic and organic nitrogen sources (Cochlan et al., 2008; Howard et al., 2007); organic matter, including urea, seem to have a positive effect on the growth of *P. delicatissima* (Loureiro et al., in press); DMSP (dimethylsulfoniopropionate) sulphur was incorporated and assimilated by *Pseudo-nitzschia* sp. (Vila-Costa et al., 2006).

The Ebro River, after a course of 928 km, flows into the NW Mediterranean Sea forming the Ebro Delta (320 km²), one of the most important wetlands from the western Mediterranean. Part of this area is classified as a Natural Park, a Ramsar wetland and a Natura 2000 European site. The main anthropogenic influences in the Delta comprehend intensive agricultural practices (65% of the total surface), urban and industrial discharges, fisheries,

aquaculture and tourism activities (Gómez-Gutiérrez et al., 2006; Prat and Ibáñez, 1995). Included in this area is the Alfacs Bay, a semi-confined water mass in the southern edge of the Delta (Fig. 1). The bay is subject to freshwater inputs of the drainage channels from rice field cultivation, which constitute the basis of the agricultural activities within the Delta (Claver et al., 2006; Gómez-Gutiérrez et al., 2006; Prat et al., 1988). The inputs of inorganic nutrients along with benthic remineralization processes are considered key factors in the occurrence of microalgae blooms in Alfacs Bay (Delgado and Camp, 1987; Prat et al., 1988). However, the potential influence of the presence of dissolved organic nutrients in the development and sustenance of such blooms has not yet been addressed in this location.

Several harmful algae species occur in Alfacs Bay including *Alexandrium minutum*, *Dinophysis* spp., *Prorocentrum lima*, *Karlodinium veneficum* and *K. armiger* (formerly identified as *Gyrodinium corsicum*), responsible for Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP) and ichthyotoxic events (Cembella et al., 2005; Delgado et al., 1990; Garcés et al., 1999) respectively. This area is additionally subject to recurrent blooms of *Pseudo-nitzschia* spp.. Blooms prevail for several months often exceeding abundances of 10^6 cell L⁻¹ (Fernández-Tejedor et al., 2008). *P. calliantha*, *P. delicatissima*, *P. pungens*, *P. multistriata* and *P. fraudulenta* are among the species detected in this area, with *P. calliantha* and *P. delicatissima* as the most abundant species (Quijano-Scheggia et al., 2008a).

The general objective of this work was to study the dynamics between the dissolved organic pool and microalgae in Alfacs Bay. The specific objective was to investigate the trophic regime of *Pseudo-nitzschia* spp. in this area where blooms are a natural and recurrent phenomenon. We hypothesize that Dissolved Organic Matter (DOM) may exert an influence on the development of this potentially harmful diatom in this embayment. In order to achieve our goal (1) data on biological, physical and chemical variables, including the DOM pool,

were collected during one year (2007 - 2008) and (2) a DOM enrichment experiment was performed with field samples collected during a *Pseudo-nitzschia* spp. bloom event. Bacteria, a source and sink of DOM (Nagata, 2000) with a recognized influence on the development and decay of HAB events (Brussaard et al., 2005; Garcés et al., 2007), were also addressed in our experimental work.

MATERIAL AND METHODS

Sampling area. The Alfacs Bay has an average depth of 3.13 m and an area of 49 km², being connected to the sea by a 2 km wide inlet (Camp and Delgado, 1987). It is one of the most important areas of aquaculture practice (mussels, *Mytilus galloprovincialis*, and oysters *Crassostrea gigas*) of the NE coast of Spain. Hydrological and circulation patterns are influenced by phenomena such as coastal currents, wind stress and agricultural freshwater discharges (Camp and Delgado, 1987). The rice cultivation (April – September) imposes a periodic flooding and drainage of the rice fields, modifying the natural hydrological regime of this area (Serra et al., 2007).

Sampling and experimental design. Surface (0.5m) seawater was sampled weekly at Alfacs Bay central station (40° 36' 0" N, 0° 39' 0" E), between April 2007 and March of 2008 to complete a year cycle. Aliquots were used for the determination of chemical and biological parameters. An experiment was set with seawater from the same station, collected on the 21 September (2007), during a *Pseudo-nitzschia* spp. bloom (cell abundances of $\approx 10^6$ cell L⁻¹). The water was filtered through a 150 µm mesh to exclude large particles and predators and distributed in 2 L polycarbonate containers. The duplicate treatments consisted on: Control (seawater without nutrient addition), N+P (addition of nitrate and phosphate), DOM (addition

of DOM), (-N+P)+DOM (addition of DOM and phosphate, nitrogen deficient), (N+P)+DOM (addition of DOM, nitrate and phosphate), B+DOM (bacteria fraction with DOM addition; bacteria were removed by filtration of the seawater through 0.8 μ m Nuclepore filters).

High Molecular Weight Dissolved Organic Matter (>1000 Da, HMW DOM) was extracted from Alfacs Bay seawater and used for the enrichment of DOM assays. The water was filtered through a 0.2 μ m Pall Corporation cartridge and concentrated (factor: 50x) by tangential-flow filtration (Prep/Scale-TFF cartridge, Millipore). DOM additions were in the order of twice the original (2 x DOM) (Boyer et al., 2006), whereas nitrate and phosphate were added at 2 and 10 μ M, respectively. Silicate was not added to the experimental bottles, given that the concentration in the natural water, ca. 8 μ M, allowed a non-limiting N:Si close to 1 in the N enriched treatments (Egge and Aksnes, 1992). The experiment was run in batch mode in an outdoor incubation with continuous water flow; the bottles were gently mixed three times a day. Temperature and solar radiation were measured every 2 hours along the light period. According to the follow-up of the biotic assemblage by optical microscopy, days 0, 2 and 4 were selected for the sampling of chemical and biological variables. Days 2 and 4 corresponded to the peak and decline of the microplanktonic assemblage.

Chemical and biological analysis. Temperature and salinity were measured with a YSI 556 Multi Probe while radiation was determined by means of a Li-Cor Point Quantum Sensor (Model LI-185 B, Li-Cor, Inc., Lincoln, NE). Samples for the estimation of inorganic nutrients (ammonium, nitrite, nitrate, phosphate and silicate) were stored at -20 °C and further determined with an auto-analyzer (Alliance Evolution II) using standard colorimetric techniques (Grasshoff et al., 1983). DIN (Dissolved Inorganic Nitrogen) was computed as the sum of ammonium, nitrate and nitrite. DOM was estimated by measurements of DOC, DON and DOP (Dissolved Organic Carbon, Dissolved Organic Nitrogen and Dissolved Organic

119 Phosphorus). Samples for dissolved organic elements analyses were filtered through pre-
120 combusted (450 °C, 6 h) Whatman glass fiber filters (0.7 µm nominal pore). DOC samples
121 were fixed with H₃PO₄, stored at 4°C in 10 ml flame-sealed glass ampoules (pre-combustion:
122 450 °C, 24h) and analysed by high-temperature catalytic oxidation by means of a Shimadzu
123 TOC-V (Álvarez-Salgado and Miller, 1998). The instrument sensitivity was checked regularly
124 by analyses of low carbon and deep Sargasso Sea water samples (Certified Reference
125 Materials for Dissolved Organic Carbon Analysis) supplied by Dr Hansell and Dr W. Chen
126 (University of Miami, US). TDN (Total Dissolved Nitrogen) and TDP (Total Dissolved
127 Phosphorus) were determined using a Bran+Luebbe AA3·auto analyzer, after persulfate
128 oxidation (Grasshoff et al., 1999); DON was estimated by withdrawing DIN (Dissolved
129 Inorganic Nitrogen = nitrate + nitrite + ammonium) from TDN, and DOP was estimated by
130 subtracting the dissolved inorganic phosphate from TDP. Chlorophyll *a* (chl *a*) samples were
131 filter-extracted in acetone for 48h and analysed in a Turner 10 AU fluorometer (Yentsh and
132 Menzel, 1963). Samples for the identification and quantification of the microplanktonic
133 assemblage were maintained either in formol (field samples) or lugol-iodine solution
134 (experimental samples), sedimented (24h), and quantified with a Leica-Leitz DM-IL inverted
135 microscope in a suitable area (Andersen and Throndsen, 2003). *Pseudo-nitzschia* species were
136 identified by SEM (Scanning Electron Microscopy) according to their morphometric
137 characteristics (width and length of the valve, density of the striae, fibulae and poroids,
138 structure of the girdle bands and the perforation patterns in the poroid hymens). The organic
139 material in the samples was removed by acid treatment as in Lundholm et al. (2002).
140 Thereafter, the sample was mounted on a polycarbonate filter, attached on stubs with colloidal
141 silver and further sputter-coated with gold-palladium. The stubs were then screened by a
142 Hitachi S-3500N microscope operating at 5 kV. In each sample, 30 – 50 cells were identified,
143 depending on the species abundance and the composition of the sample. Bacterial cell

numbers were estimated in 2 mL subsamples that were fixed with 1% paraformaldehyde (final concentration), frozen in liquid-N, kept at -80° and processed with a Becton-Dickinson FACScalibur flow cytometer (Gasol and delGiorgio, 2000). After thawing, samples (500µl) were stained with Syto 13 (Molecular Probes), mixed with yellow-green latex beads (Polyscience) and run at low speed until 10.000 events were registered in a side scatter (SSC) versus green fluorescence (FL1) plot.

Statistical treatment. Non-parametric analyses were applied because most parameters were not normally distributed (Shapiro-Wilk tests). Spearman rank-order correlations (r_s) and Wilcoxon Matched Pairs Test were determined with STATISTICA[®] 6 software. Multivariate analyses were performed with PRIMER[®] 6 package software according to Clarke & Warwick (2001). The ANOSIM (analysis of similarities) routine tested for the significant differences between the a priori seasonal groups, with R as a measure of the statistical separation among the groups (range: 0 – 1). Cluster analysis was performed on the Bray-Curtis similarity matrix from the square-root transformed abundance data of the total microplanktonic assemblage. SIMPER (similarity percentages) analysis identified the most discriminating biotic and abiotic variables regarding the cluster (seasonal) groups. Abiotic parameters considered in this analysis include temperature, salinity, oxygen, silicate, phosphate, nitrate, nitrite, ammonium, DOC, DON and DOP.

RESULTS

Alfacs temporal sampling

The water temperature in Alfacs Bay reached its maximum during summer (July and August) and minimum in winter (December and January), as expected for a temperate basin, whereas salinity was lower in spring (coinciding with the period of highest freshwater supply from the rice fields) and higher in winter (with minimum freshwater input) (Fig. 2a, b; Table 1). Concerning nutrients, ammonium and nitrate were higher in spring and winter time (Table 1). Silicate displayed several peaks during the year (June, August, October and December) with a decreasing trend in winter (Fig. 2c). DIN reached maximum values in April ($23.4 \pm 8.8 \mu\text{M}$) and December – February ($13.1 \pm 0.5 \mu\text{M}$), followed by lower concentrations during the remaining time of the study, whereas the nitrogen organic pool (DON) generally followed a reverse pattern with highest levels from June – December (maximum: $33 \mu\text{M}$, November) (Fig. 2d). DON was significantly higher than DIN (Wilcoxon test, $p < 0.006$) during the summer and fall. As regards phosphorus contents, phosphate concentrations varied between $0.14 \mu\text{M}$ (June and July) and $0.23 \mu\text{M}$ (April), while DOP had a gradual increase from April until August (maximum: $1.2 \mu\text{M}$, August), and a decreasing trend thereafter (Fig. 2e). DOP summer concentrations were significantly higher than those of phosphate (Wilcoxon test, $p = 0.003$). DOC peaked during July (maximum: $356 \mu\text{M}$, November) (Fig. 2f). The salinity was inversely correlated with nitrate ($p < 0.05$, $r_s = -0.62$) and DOP ($p < 0.05$, $r_s = -0.65$) during the summer, with DON during the fall ($p < 0.05$, $r_s = -0.73$), and with all inorganic nutrients ($p < 0.05$, $r_s > -0.65$), except phosphate, in the winter; during the spring no significant correlation was found between salinity and nutrients.

Chlorophyll *a* concentration, herein as a proxy of the microalgae biomass, attained higher mean values in the spring and fall (Fig. 3a). Regarding microalgae groups, diatoms prevailed over the remaining components of the microplankton assemblage, except during December, January and March when the community was dominated by other phytoplankton groups (coccolithophorids, cyanobacteria and nanoflagellates) (Fig 3b). Conversely,

dinoflagellates had a low contribution to the total assemblage with a maximum contribution in June (when it accounted for 20% of the total phytoplankton cell abundance). Diatoms were negatively associated with temperature and silicate in spring and with nitrate, silicate, DOP and salinity in winter (Table 2). Furthermore, there was a significant positive correlation between diatoms and chl *a* during spring and winter seasons ($p < 0.05$, $r_s > 0.75$). *Pseudo-nitzschia* spp. was the most frequent potentially harmful algae observed (present in 78% of the total samples) and an important contributor to the total microplanktonic assemblage being more abundant during the fall (Fig. 3c). During spring, this species was inversely associated with temperature, DOC and DON, and directly associated with nitrate; in the summer they were positively associated with phosphate and negatively with silicate. *Pseudo-nitzschia* spp. abundances were additionally correlated with chl *a* concentration in the summer ($p < 0.05$, $r_s = 0.74$), and with total diatom numbers in the summer, fall and winter ($p < 0.05$, $r_s > 0.75$).

Statistical analysis evidenced seasonal differences in the microplanktonic structure of the Alfacs Bay (ANOSIM test, global $R = 0.4$). Furthermore, at a similarity coefficient of 40%, the dendrogram obtained by cluster analysis allowed us to identify winter as the most well-defined seasonal group, followed by fall, whereas spring samples split into two basic groups and summer samples were distributed along distinct clusters (Fig. 4). *Pseudo-nitzschia* spp. was consistently present among the species contributing the most to the dissimilarities between the seasonal clusters (SIMPER analysis), denoting its importance to the total microplankton assemblage. Additionally, DON, nitrate and silicate were the most significant abiotic variables responsible for the differentiation between the distinct seasons (cumulative contribution: 55 ± 8 %, SIMPER analysis).

DOM enrichment experiment

The natural assemblage used for the experiment was composed by 81% (in cell numbers) of *Pseudo-nitzschia* spp., 5% of other diatom species, and the remainder by dinoflagellates, nanoflagellates and ciliates. Therefore, our investigation focused mainly on the evolution of *Pseudo-nitzschia* cells. Chlorophyll *a* gradually declined from day 0 to day 4 in most treatments except in (N+P) and (N+P)+DOM where chl *a* peaked on day 2 (Fig. 5a). *Pseudo-nitzschia* cell abundance had a more scatter variation (Fig. 5b). Interestingly, unlike other treatments, *Pseudo-nitzschia* cells of DOM bioassay reached a maximum concentration by the end of the experiment (day 4). This increment was not coincident with a chl *a* increase. As identified by SEM (Fig. 6), at the beginning of the experiment (day 0), *P. delicatissima*, *P. brasiliensis* and *P. calliantha* accounted for 7%, 29% and 64%, respectively, of the total population of *Pseudo-nitzschia*. *P. calliantha* was the predominant species along the experiment (Fig. 5c). Bacteria abundance had a small variation during this investigation (Fig. 7).

The temperature remained mostly stable throughout the incubation period (23.4 ± 0.4 °C). In regard to solar radiation, values were generally constant during the time of the experiment ($537 \pm 115 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) except for day 3 when maximum values were reached ($1930 \pm 1072 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) (data not shown). For most incubation sets, ammonium decreased in day 2, after which an accumulation was evident at the end of the experiment, exceptions being met by the inorganic enrichment treatment (N+P) and in the bacteria control (B+DOM) (Fig. 8a). Nitrate generally followed a decreasing pattern in every incubation experiment (Fig 8b), whereas phosphate followed a decrease-accumulation pattern in samples enriched with P, while it remained mostly stable in the rest of the bioassays (Fig 8c). Silicate had a declining trend over time (although it was never exhausted), except in B+DOM control where less variation was observed (Fig 8d); the fact that silicate minimum was $3 \mu\text{M}$ confirms non-limitation conditions, as expected. The dissolved organic matter

extracted from Alfacs Bay and further used for DOM enriched bioassays had a C:N = 15. Concerning the changes in the dissolved organic nutrients, DOC concentration remained generally constant during the experiment (Fig. 9a). Similarly, DON concentration showed no marked changes (Fig. 9b). DOP had a moderate increase in day 2 in the Control incubation, whereas a gradual increase until day 4 was recorded in N+P (Fig. 9c). In the DOM and the (N+P)+DOM sets, DOP declined steadily, while in the B+DOM treatment an initial decrease by day 2 was followed by a small accumulation at the end of the experiment. In the (-N+P)+DOM, there was no variation at DOP concentration.

DISCUSSION

Alfacs temporal study

Temperate estuaries and coastal lagoons are generally characterised by high salinity levels during the summer season because of minimum precipitation and high evaporation phenomena. However, concurrent with previous works (Camp and Delgado, 1987; Comin et al., 1987), the salinity in Alfacs Bay was found to deviate from standard patterns because of the periodic freshwater discharges from the rice fields that keep values at lower levels during most year. Precipitation was not correlated to salinity (data not displayed), corroborating the importance of the freshwater inflow from agriculture activities to the salinity level in this embayment. Statistical inverse correlations between salinity and nutrients reflect the input of allochthonous trophic material into the bay during the period of freshwater discharges (Delgado & Camp 1987). The disruption of the close association between salinity and

nutrients in the spring suggests that during this season other factors (e.g. autotrophic uptake from the spring blooms) were equally important for the dynamics of the nutrient pool.

Inorganic nutrient concentrations were within the range of reported values for this location (Quijano-Scheggia et al., 2008a). Although dissolved inorganic nutrient concentrations in the embayment were higher than in the adjacent coastal shelf (Arin et al., 2005), values were low as compared with eutrophic bays (Puigserver et al., 2002). Indeed, a decreasing trend in inorganic nutrient loadings, specially regarding phosphorus, has recently been observed at the lower Ebro River (Ibáñez et al., 2008; Sierra et al., 2002). The content of inorganic nutrient levels observed during winter when the flux of nutrients from freshwater supply was minimum is likely the result of biotic processes as remineralisation (Delgado and Camp, 1987) and decreased sequestering by the lower autotrophic biomass. The remineralisation of organic matter is acknowledged as a significant source of inorganic nutrients in coastal systems (Nixon, 1981).

The fact that the gradual increase in dissolved organic N (from 4% in April to 78% in August) and P (from 35% in April to 84% in August) in regards to the total dissolved nutrients was coincident with the opening of the freshwater channels (spring), suggests an important contribution of organic nutrients within these discharges. DOC concentrations concur with previous data for this area (Gómez-Gutiérrez et al., 2006) and are within the range of similar shallow embayments (e.g. Chesapeake bay 118 – 215 μ M) (Minor et al., 2006). The summer maximum was feasibly associated with the accumulation of DOC from allochthonous sources as well as autochthonous material originating from the previous spring bloom. A lagged post-bloom increase of DOC concentration was already reported in several occasions (Wafar et al., 1984 and ref therein). Auto-lysis, degradation and exudation processes are among the processes responsible for this increment. DON was generally lower than the common levels of estuarine and river systems but in the same order of magnitude as

coastal ecosystems (Berman and Bronk, 2003, ref. therein). The highest concentrations were attained in November and December when the freshwater discharges from the rice fields were at their minimum. Although there was no close association with rain events, increased DON concentrations coincided with periods of strong wind conditions ($> 11 \text{ m s}^{-1}$) (data not displayed) and variation in salinity surface levels. These features may point to a vertical mixing, resuspension of deposited material and uniformization of water layers that otherwise remain separated by salinity stratification (Camp and Delgado, 1987). Furthermore, the decrease of freshwater flux from agriculture activities in this period contributes to a longer retention time of the water within the bay reducing the export to coastal waters. Therefore, we hypothesize that DON peaks during late fall may have been related to the new availability and further retention of organic material in surface waters through physical forcing mechanisms. DON could have originated from autochthonous events such as exudation and grazing processes related to the fall phytoplankton bloom. DOP higher levels were coincident with the peak in freshwater discharges (May to September), which may reflect the main allochthonous nature of this organic pool. Although inorganic N:P ratios were generally higher than 16 (48 ± 37) the inference of actual phosphorus limitation by microalgae in such conditions would be too simplistic. Aside from (1) possible inherent analytic errors (2) the fluctuation of ratios by nutrient loadings (3) the problematic evaluation of the availability of elements to the phytoplankton community (4) the presence of intercellular pool of nutrients and (5) the plasticity of the Redfield ratio of microalgae (Geider and LaRoche, 2002; Hecky et al., 1993), there is growing evidence of the use of organic phosphorus by microalgae, including diatoms (Diaz et al., 2008; Yamaguchi et al., 2005), the greater components of the microalgae community in Alfacs Bay. If this is to be the case, inorganic phosphorus limitation could be obviated by the uptake of organic phosphorus components, at least in some organisms.

The sequential appearance of maximums within the DOM seasonal cycle has been related to the seasonal changes of C:N:P ratios and the distinct processing rates of these elements (production and decomposition) (Wafar et al., 1984). However, Wafar et al. (1984) investigation was associated with a permanent well-mixed water body that does not match the characteristics of the system under study. The temporal sequence of organic maximums (DOC in July, DOP in August and DON in November) in a complex system such as the Alfacs Bay, in which an array of abiotic and biotic conditions of allochthonous and autochthonous nature continuously succeed and interact, is not likely a linear consequence of dominating variables but rather an effect of context dynamics between physical, chemical and biological factors.

Chlorophyll *a*, although lower than in eutrophic areas was within the range of Mediterranean bays (Balkis, in press; Polat, 2002). Summer concentrations were lower than found by Quijano-Scheggia (2008a) for the same location, reflecting a interannual variability of parameters common to dynamic coastal systems. The seasonal variation of chl *a* and nutrient statistical relations agrees with a seasonal switch in nutrient patterns as observed in coastal transition areas such as estuaries, lagoons and embayments (Fong et al., 1993; Malone et al., 1996). Additionally, the association between chl *a* and organic material points to a link between these parameters. In Alfacs bay, where inorganic substrates were surpassed by their organic counterparts during most of the year, the use of the organic pool by phytoplankton would constitute an advantageous nutritional strategy to counteract possible inorganic nutrient limitation conditions. Indeed, mixotrophy seems to be more frequent than previously recognized (Troost et al., 2005) playing a significant role within natural microalgae communities (Klug, 2002; Stolte et al., 2006).

The microplankton assemblage was generally grouped according to the sampling season. Although salinity is recognised as an important variable influencing the physical and chemical dynamics within the Alfacs bay (Camp and Delgado, 1987), chemical parameters as

silicate, DON and nitrate were identified by multivariate routine as the most significant abiotic parameters responsible for the inter-seasonal differentiation. The considerable contribution of diatoms within the total microalgae assemblage in this location corroborates with the importance of silicate in this analysis. These outcomes also point to DON as a relevant factor that may influence the seasonal structure of the microalgae assemblage. Accordingly, it is acknowledged that most of the nitrogen fixed in the aquatic environment is in the form of dissolved organic material (Bronk, 2002) and that DON represents an active nitrogen resource for phytoplankton in the aquatic realm (Berman and Bronk, 2003).

Within the diatom group, *Pseudo-nitzschia* spp. represented the most abundant genus as already reported (Quijano-Scheggia et al., 2008a). The importance of this species to the total microalgae assemblage was confirmed by multivariate statistical analysis. Such is not a random event because recurrent blooms of *Pseudo-nitzschia* have been registered recently throughout the Mediterranean coast (Caroppo et al., 2005; Quijano-Scheggia et al., 2008b; Spatharis et al., 2007). The wide blooming season of *Pseudo-nitzschia* ($>10^5$ cell L⁻¹, July – November) in Alfacs has been explained by the succession of distinct species able to adapt to the high variability of abiotic and biotic conditions present in this embayment (Quijano-Scheggia et al., 2008a). Recent studies suggest organic nutrients as a factor that may contribute to the extended window of time of *Pseudo-nitzschia* proliferations (Cochlan et al., 2008; Loureiro et al., in press). In this study, we found an association between organic nitrogen and *Pseudo-nitzschia* spp. cell abundance, which could have implications in the frequent proliferations of this species in Alfacs Bay. This issue was further addressed by performing a bioassay with natural samples during a *Pseudo-nitzschia* bloom.

Experimental work during a *Pseudo-nitzschia* spp. bloom

Considering nitrogen mass balance calculations (Parsons et al., 1961), the build-up of autotrophic biomass (chl *a*) by day 2 in N+P and (N+P)+DOM incubations may be justified by the uptake of inorganic nitrogen sources ($1\ \mu\text{mol N} \approx 1 - 4\ \mu\text{g chl } a$). The decrease in chl *a* from day 2 to day 4 indicates that the microalgae assemblage in N+P and (N+P)+DOM incubations reached the declining stage of batch growth. Furthermore, high irradiance levels, as registered during day 3, could have been associated to the reduction of photosynthetic pigments (MacIntyre et al., 2002). However, we expect a minor contribution of photoacclimation mechanisms in the sharp decline of chlorophyll content observed during this experiment (4 – 5 fold) because of the short-time of high irradiance exposure ($\approx 6\ \text{h}$). In experiments performed with *Skeletonema costatum*, the chlorophyll content took three days to decrease 2 fold at $1200\ \mu\text{mol photon m}^{-2}\ \text{s}^{-1}$, with a fast recovery of cells upon return to a low light regime (Anning et al., 2000). Additionally, diatoms, the major contributors to the total microalgae assemblage herein, are considered more flexible to variations in light regime over other microalgae groups (Wagner et al., 2006). The fact that in Control and N-deficient treatment [(-N+P)+DOM] there was no visible increment in chl *a* concentration suggests a N-limited microalgae assemblage at the time of the experiment.

The results obtained regarding DOM assay deviates from the regular positive relation between chl *a* and phytoplankton. In this case, the increase in *Pseudo-nitzschia* cells (day 4), specifically *P. calliantha*, was not accompanied by an increase in chlorophyll *a*. In recent investigations exploring the influence of the organic pool in a strain of *P. delicatissima* development, it was observed that N-deficient cultures supplemented with DOM kept an exponential growth rate similar to cells grown in nutrient-sufficient growth medium, whereas the chlorophyll cell content decreased (Loureiro et al., in press). This was explained by both nitrogen limitation, whereby N-containing molecules – as chlorophyll - decrease, and potential mixotrophic growth, whereby the acquisition of nitrogen through the DOM fraction

would reduce the need of photosynthetic pigments. In our investigation, the time-lag in *P. calliantha* growth in DOM treatment, as opposed to the fast increase of cells in N+P and [(N+P)+DOM] incubations, could point to an adaptation period to a new substrate resource. An identical delay in the onset of *P. multiseriis* cells grown under an organic nutrient regime was related to the switch of nutritional strategies, reflecting as such the adjustment of cells to new trophic conditions (Mengelt and Prézelin, 2002). Also, DOM photochemical degradation mechanisms after day 3 (corresponding to the maximum of irradiance levels) may have rendered the organic pool more biologically available favouring microalgae uptake (Moran and Zepp, 1997). Concerning the nutrient dynamics involved in DOM bioassay, a decrease in organic matter concentrations was only evident for DOP. However, the absence of a decrease in DOC and DON concentrations does not discard the uptake of organic molecules since the growth of multispecific assemblages in batch mode should cause a visible increase of organic material originating in biological processes as autotrophic exudation, viral lysis, excretion and “sloopy feeding” by grazers (Nagata, 2000). The fact that such an increase was not observed points to a fast coupling between production and consumption mechanisms (Bronk et al., 2007). Bacteria were mostly constant in DOM treatment and remaining incubations suggesting a control of these populations by mechanisms as viral lysis (Weinbauer, 2004) and grazing pressure (Vaqué et al., 1994).

Main conclusions

During the time of this investigation, the seasonal cycle of DOM was characterised by the sequential appearance of DOC (July), DOP (August) and DON (November) maximums. Within the total dissolved nutrients, the organic pool was higher than the inorganic fraction during most of the year. The microplanktonic assemblage was mainly composed by diatom

life-forms, with *Pseudo-nitzschia* spp. as the most abundant genus and frequent harmful algae. The wide temporal blooming period of *Pseudo-nitzschia* (May – November) reflected the high plasticity of the distinct species within this genus. In regards with the analysed abiotic parameters, DON, nitrate and silicate were the most significant variables contributing to the dissimilarities between seasons and thereby potentially influencing the seasonal structure of the microalgae assemblage during the period of study. This and the higher contents of the N organic fraction suggest that DON may represent a significant nutrient resource in this embayment. With regard to the experimental study, the increase of *P. calliantha* cells by the end of the experiment (day 4) could be associated with nutrient acquisition mechanism through the DOM fraction that may have induced a pigment-sparing downregulation of chlorophyll *a*.

Overall, field and experimental data point to the importance of DOM in the nutrition of the microalgae assemblage in the Alfacs bay. *Pseudo-nitzschia* spp., as the largest contributor to this assemblage, seems to be influenced by the presence of the organic pool in this embayment. This adds to the growing information regarding the potential significance of organic nutrition in the growth of *Pseudo-nitzschia* spp.

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644

645 Table 1. Means $\pm$ SD of relevant biotic and abiotic parameters from Alfacs Bay central station during each season and all year round (April 2007
646 – March 2008).

	Spring	Summer	Fall	Winter	Year	647
Temperature (°C)	19.5 $\pm$ 3.9	25.6 $\pm$ 1.0	17.2 $\pm$ 4.2	12.2 $\pm$ 1.4	18.8 $\pm$ 5.7	648
Salinity	32.5 $\pm$ 3.2	33.7 $\pm$ 0.9	33.1 $\pm$ 0.6	34.6 $\pm$ 1.1	33.5 $\pm$ 1.9	649
Ammonium (μ M)	2.31 $\pm$ 0.24	1.13 $\pm$ 0.52	1.46 $\pm$ 1.18	1.55 $\pm$ 0.59	1.61 $\pm$ 0.76	651
Nitrate (μ M)	9.22 $\pm$ 9.92	2.37 $\pm$ 0.68	5.87 $\pm$ 3.45	8.40 $\pm$ 4.26	6.46 $\pm$ 5.59	652
Silicate (μ M)	5.79 $\pm$ 5.35	7.19 $\pm$ 3.26	10.12 $\pm$ 2.08	4.47 $\pm$ 2.07	6.89 $\pm$ 3.67	653
DIN (μ M)	13.80 $\pm$ 12.90	4.08 $\pm$ 1.74	4.84 $\pm$ 2.88	12.00 $\pm$ 5.50	8.60 $\pm$ 8.30	654
Phosphate (μ M)	0.19 $\pm$ 0.04	0.16 $\pm$ 0.02	0.17 $\pm$ 0.02	0.18 $\pm$ 0.01	0.17 $\pm$ 0.02	655
DOC (μ M)	171 $\pm$ 50	232 $\pm$ 68	187 $\pm$ 37	181 $\pm$ 79	192 $\pm$ 63	656
DON (μ M)	2.98 $\pm$ 3.74	10.25 $\pm$ 3.44	15.33 $\pm$ 8.68	6.30 $\pm$ 7.80	8.60 $\pm$ 7.70	657
DOP (μ M)	0.31 $\pm$ 0.25	0.61 $\pm$ 0.33	0.22 $\pm$ 0.17	0.08 $\pm$ 0.11	0.30 $\pm$ 0.30	658
Chl <i>a</i> (μ g L ⁻¹)	3.25 $\pm$ 1.38	2.30 $\pm$ 0.71	3.87 $\pm$ 1.87	2.32 $\pm$ 0.84	2.90 $\pm$ 1.40	659
Diatoms (cell L ⁻¹)	8.90 10 ⁴ $\pm$ 1.25 10 ⁵	3.76 10 ⁵ $\pm$ 3.23 10 ⁵	3.72 10 ⁵ $\pm$ 3.42 10 ⁵	8.82 10 ⁴ $\pm$ 1.35 10 ⁵	2.32 10 ⁵ $\pm$ 2.83 10 ⁵	660
Dinoflg. (cells L ⁻¹)	1.28 10 ⁴ $\pm$ 8.37 10 ³	2.68 10 ⁴ $\pm$ 2.85 10 ⁴	2.94 10 ³ $\pm$ 1.22 10 ³	4.88 10 ³ $\pm$ 2.66 10 ³	1.24 10 ⁴ $\pm$ 1.77 10 ⁴	661
Psn (cells L ⁻¹)	2.30 10 ⁴ $\pm$ 3.66 10 ⁴	2.64 10 ⁵ $\pm$ 3.47 10 ⁵	3.31 10 ⁵ $\pm$ 3.40 10 ⁵	1.55 10 ⁴ $\pm$ 1.65 10 ⁴	1.58 10 ⁵ $\pm$ 2.75 10 ⁵	662
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672 Dinoflg. = dinoflagellates; Psn = *Pseudo-nitzschia* spp.

673 Table 2. Spearman rank-correlations ($p < 0.05$) between biotic and abiotic parameters at Alfacs Bay (April 2007 – March 2008); Psn = *Pseudo-*
674 *nitzschia* spp.; Diat = diatoms; Dino = dinoflagellates; Spr = spring; Sum = summer; Fal = fall; Win = winter.
675

	Chla				Diat				Psn			
	Spr	Sum	Fal	Win	Spr	Sum	Fal	Win	Spr	Sum	Fal	Win
T	*+				*+				*+			
Sal				++				+				
PO ₄		+								+		
NH ₄	++		*++									
NO ₃				*++				*+	+			
SiO ₄	*+	*+	*+	*++	*++			*++		*+		
DOC	*++	*+							*+			
DON	*+								*+			
DOP								*+				

676 * denotes an inverse correlation; + stands for correlations $> 0.50, < 0.75$; ++ stands for correlations > 0.75 .

677 Fig. 1. Geographic location of the sampling station (black dot) (40° 36' 0" N, 0° 39' 0" E) in the Alfacs Bay, Ebro Delta (NW Mediterranean).
678

679 Fig.2. Monthly means of temperature (a), salinity (b), silicate (c), TDN (Total Dissolved Nitrogen) that include DIN (Dissolved Inorganic
680 Nitrogen) and DON (Dissolved Organic Nitrogen) (d), TDP (Total Dissolved Phosphorus) that include phosphate and DOP (Dissolved Organic
681 Phosphorus) (e), DOC (Dissolved Organic Carbon) (f). Error bars correspond to standard deviations; dashed lines divide the plots into distinct
682 seasons (spring, summer, fall and winter).

683

684 Fig. 3. Temporal changes in the chlorophyll *a* concentration (a), relative cell abundances (in %) of the main groups within the microplankton
685 assemblage (b), and *Pseudo-nitzschia* spp. cell numbers temporal evolution (c) over the studied period. Diat = diatoms; Psn = *Pseudo-nitzschia*
686 spp.; dino = dinoflagellates; other = coccolithophorids + cyanobacteria + nanoflagellates; error bars correspond to standard deviation; dashed lines
687 divide the plots according to the distinct seasons (spring, summer, fall and winter).

688

689 Fig. 4. Dendrogram generated by the cluster analysis of the Bray-Curtis similarity matrix of the microplankton abundance data (square-root
690 transformed). Seasons: spr = spring, sum = summer, fal = fall, win = winter.

691

692 Fig. 5. Chlorophyll *a* (a), *Pseudo-nitzschia* spp. (b) and *P. delicatissima*, *P. brasiliiana* and *P. calliantha* evolution during the experiment. Error
693 bars correspond to standard deviations.

694

695 Fig. 6. SEM (Scanning Electron Microscopy) images of *Pseudo-nitzschia* species identified during the enrichment experiment. A, *P.*
696 *delicatissima*; B, *P. brasiliiana*; C, *P. calliantha*.

697

698 Fig. 7. Bacteria abundance during the enrichment experiment, as estimated by flow cytometry. Error bars correspond to standard deviations.

699

700 Fig. 8. Ammonium (a), nitrate (b), phosphate (c) and silicate (d) concentrations at days 0, 2 and 4 of the DOM enrichment experiment. Error bars
701 correspond to standard deviations.

702

703 Fig. 9. DOC (a), DON (b) and DOP (c) concentrations at days 0, 2 and 4. Error bars correspond to standard deviations.